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Inhibitory, GABAergic Nerve Terminals Decrease at Sites of Focal Epilepsy

Abstract. *Using an immunocytochemical method for the localization of the γ-aminobutyric acid (GABA) synthesizing enzyme, glutamic acid decarboxylase (GAD), we have observed GABAergic nerve terminals distributed throughout all layers of normal monkey sensorimotor cortex. These terminals displayed ultrastructural characteristics that suggested that they arose from aspinous and sparsely spinous stellate neurons. In monkeys (*Macaca mulatta *and* M. fascicularis*) made epileptic by cortical application of alumina gel, a highly significant numerical decrease of GAD-positive nerve terminals occurred at sites of seizure foci indicating a functional loss of GABAergic inhibitory synapses. A loss of such inhibition at seizure foci could lead to epileptic activity of cortical pyramidal neurons.*

Epilepsy is a neurological disorder characterized by intermittent, generalized seizures involving motor and sensory systems. Most epilepsies in humans are caused by tumors or by trauma to a brain region as a result of cranial injuries, including those produced by birth canal obstructions (*1*). Human epilepsy has not been studied in as much detail as disorders in experimental animals produced by the application of various agents that initiate seizure activity; for example, alumina gel, cobalt, and penicillin (*2*). Since these experimentally produced epileptic foci develop reproducibly they have provided models for the study of human focal epilepsy.

Several factors, including glial hypertrophy, ischemia, and increased concentrations of acetylcholine, have been suggested as being involved in the onset of seizures (*3*). Another such factor, and one that is the concern of this report, involves the inhibitory neurotransmitter γ-aminobutyric acid (GABA). It has been suggested that a reduction of this inhibitory substance could be responsible for seizure activity because results from biochemical studies have shown decreases in both GABA (*4*) and its synthesizing enzyme, glutamic acid decarboxylase (GAD) (*5*), at seizure foci.

In a recent immunocytochemical study, GAD has been localized within the axon terminals of aspinous and sparsely spinous stellate neurons in the rat cerebral cortex (*6*). Since GAD has been found in a number of other brain regions within neurons that have been identified as GABAergic (*7*), it is probable that the presence of GAD is indicative of neurons that use GABA as a neurotransmitter. Therefore, the localization of GAD within aspinous and sparsely spinous stellate neurons, together with evidence from physiological and pharmacological studies (*8*), strongly suggests that these neurons are responsible for GABA-mediated inhibition in the cerebral cortex. Since these neurons are found in every cortical layer and project numerous axon terminals to pyramidal cell somata, they could exert a powerful inhibitory effect on cortical projection neurons. Furthermore, a decrease in the number of these inhibitory axon terminals could lead to seizure activity of pyramidal neurons. To test this possibility, we compared the

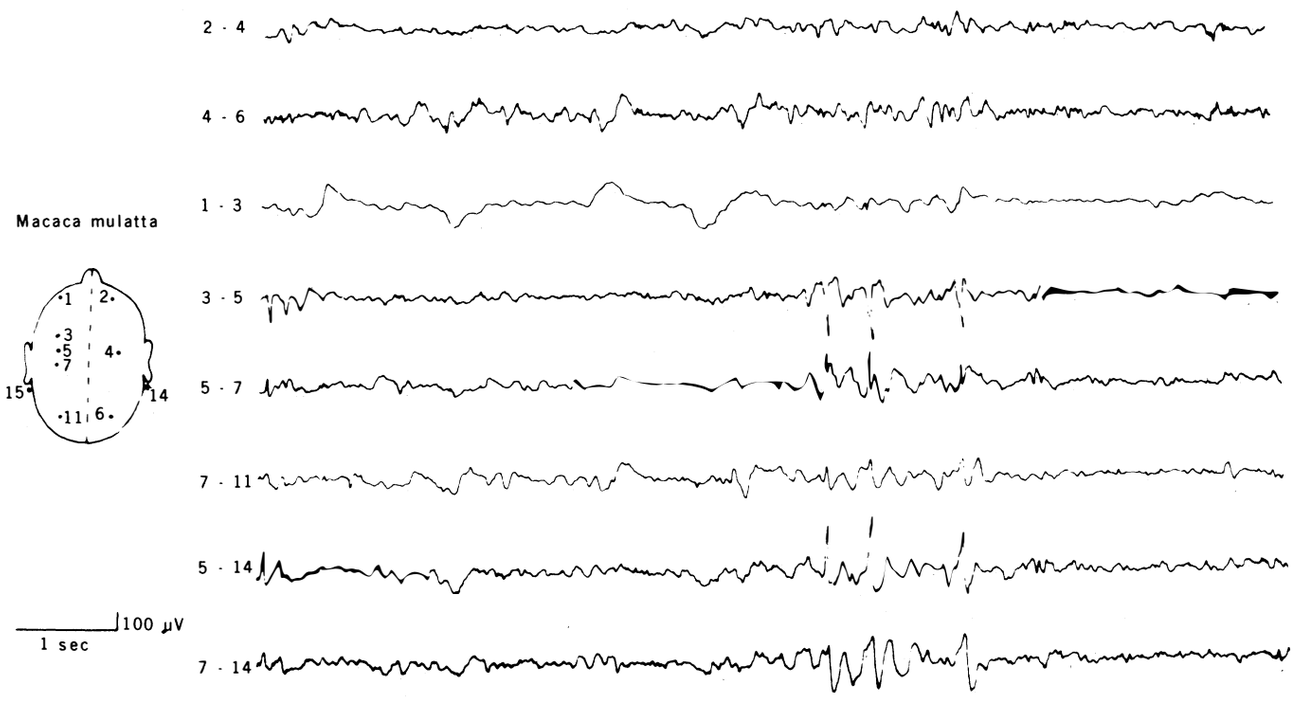

Fig. 1. An ECG recording that was made just prior to fixation of the brain by intravascular perfusion shows an alumina-induced epileptic focus in *M. mulatta* located around lead 5 as indicated by spike phase reversals in traces 3 to 5 and 5 to 7.

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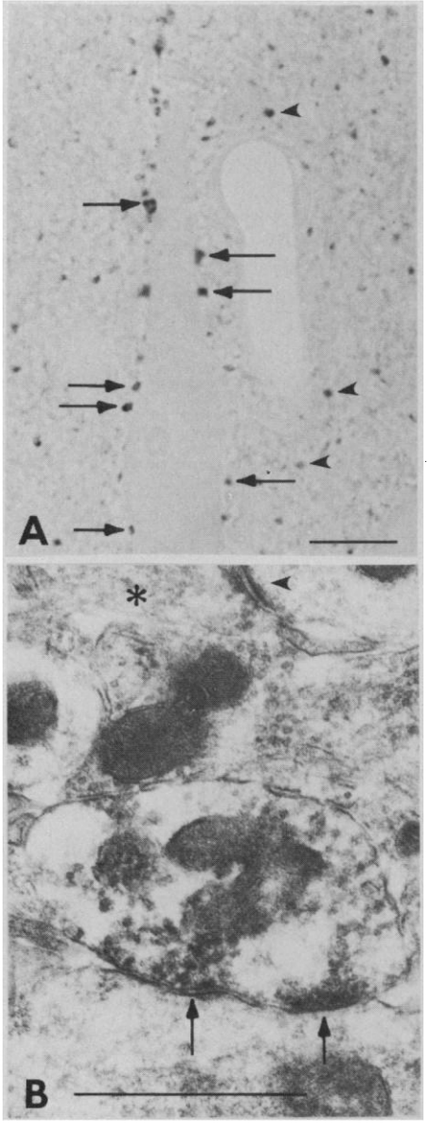
numbers of GAD-positive axon terminals in homotopic, nonepileptic sensorimotor cortex with those found in epileptic cortex from monkeys treated with alumina gel. Our study shows a significant decrease of GAD-positive (GABAergic) terminals at sites of seizure foci, and therefore we suggest that such a decrease could be responsible for the observed epileptic activity.

Six adult monkeys (*Macaca mulatta* and *M. fascicularis*) were used in this study. Five of the monkeys received alumina gel applications to the left cerebral hemispheres in order to produce seizure foci. Three of the five received injections directly into both pre- and postcentral gyri, and the remaining two experimental monkeys had injections of alumina gel limited to the subarachnoid space in the area of the central sulcus. In these last two monkeys, the parenchyma of the cerebral cortex was not invaded by alumina gel (*9*). Electroencephalogram (EEG) tracings from all five experimental monkeys were normal initially, but changed as epilepsy developed (*10*). Electrocorticography (ECG) of all experimental animals verified epileptic foci (*10*) (Fig. 1), and subsequently the monkeys were fixed by intracardiac perfusions of aldehydes (*11*). Blocks of tissue from epileptic cortex, from a homologous area in the contralateral nonepileptic cortex, and from comparable areas of normal cortex were processed for light and electron microscopy according to procedures described for GAD immunocytochemistry (*11*).

In addition to routine microscopic examination of 30-μm-thick sections of tissue incubated in antiserum to GAD, we counted the numbers of GAD-positive axon terminals through the entire section thickness of 38 contiguous 100-μm^2 areas



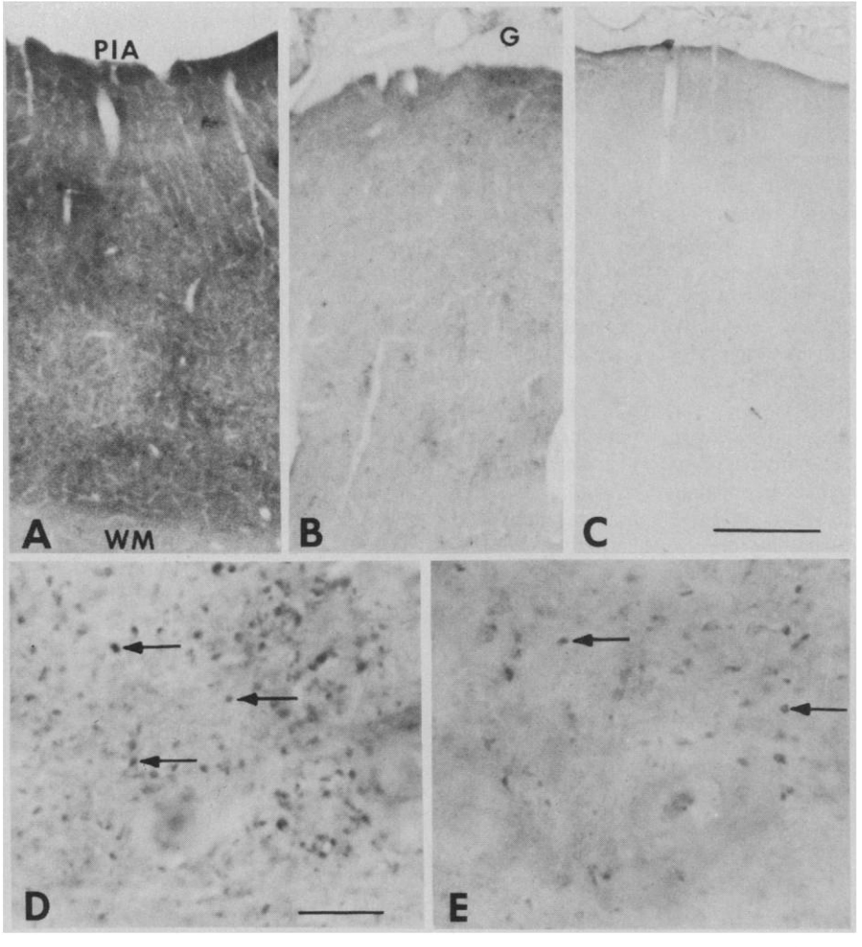


Fig. 2 (left). Normal monkey cerebral cortex incubated in antiserum to GAD. (A) Photomicrograph of a semithin (1 μm) section showing GAD-positive axon terminals (arrows) on the somal and dendritic surfaces of a layer III pyramidal neuron. Other GAD-positive terminals (arrowheads) are scattered throughout the neuropil. Scale bar, 10 μm. (B) Electron micrograph of an axon terminal containing GAD-positive reaction product around synaptic vesicles. This terminal forms a symmetric axosomatic synapse (arrows), while a GAD-negative terminal (asterisk) forms an asymmetric axodendritic synapse (arrowhead). Scale bar, 1 μm. Fig. 3 (right). Photomicrographs of sensorimotor cortex from epileptic monkeys. (A) A section from nonepileptic cortex contralateral to alumina gel application contains dense deposits of GAD-positive reaction product distributed between the pial surface (*PIA*) and the white matter (*WM*). (B) A section from the site of alumina gel application displays lower staining intensities for GAD than the section from the nonepileptic contralateral cortex (A). Alumina gel (*G*) is on the surface of the pia matter. (C) A section of cortex, incubated in control (nonimmune) serum, from the site of alumina gel application. This section does not display axon terminals, but shows a weak endogenous peroxidase-like reaction product in layers I and II that is due to peroxisomes within macrophages (see text). All three photomicrographs were matched for background gray scale in order to show actual differences in intensities of GAD-positive staining between sections. Scale bar for (A), (B), and (C), 500 μm. (D and E) Photomicrographs of sections of cerebral cortex incubated in antiserum to GAD showing the magnification (×1000) at which counts of terminals were made (see text). (D) Layer VI of nonepileptic cortex contralateral to alumina gel application shows a large number of GAD-positive terminals (arrows) in the neuropil. (E) Layer VI of cortex beneath alumina gel displays fewer GAD-positive terminals (arrows) than contralateral cortex (D). Scale bar for (D) and (E), 10 μm.

from the bottom of cortical layer VI to the upper and middle parts of layer V. For each monkey, these counts were made at equivalent locations (for example, base of central sulcus) from each of the following sites: (i) proximal to the site of alumina gel application, (ii) ipsilateral to, but farther away from the alumina gel, and (iii) contralateral, homotopic nonepileptic cortex. The first counting site was located either beneath the placement of alumina gel (for the monkeys with subarachnoid injections) or close to, but not contacting the alumina granuloma (*10*) (for the monkeys that received intracortical injections). In addition, counts were made at comparable locations in both cerebral hemispheres of a normal monkey.

In preparations from normal monkey cortex incubated in antiserum to GAD, GAD-positive reaction product appeared throughout all layers of sensorimotor cortex in the form of punctate structures (0.5 to 1.5 μm in diameter) that correspond with axon terminals (*6*, *7*). The GAD-positive axon terminals in normal monkey cortex were distributed evenly throughout the neuropil and on the surfaces of neuronal somata and dendrites (Fig. 2). The mean number of GAD-positive terminals per 3000 μm^3 of tissue sampled in layers V and VI did not differ significantly between the two normal hemispheres nor between different cytoarchitectural areas in sensorimotor cortex. The mean number of these terminals in normal pre- and postcentral gyri and in normal cortex subjacent to the central sulcus, ranged from 16 to 24 per 3000 μm^3. This same range of values was also observed in the nonepileptic hemispheres (*12*) of most experimental monkeys (see Table 1). No stained axon terminals were observed in control preparations incubated in nonimmune rabbit serum.

Preparations of normal monkey sensorimotor cortex examined by electron microscopy displayed GAD-positive reaction product within axon terminals throughout all cortical layers, and these terminals formed symmetric synaptic junctions with somata and dendrites (Fig. 2). Since electron microscopic studies of Golgi-stained neurons indicate that cortical terminals forming this type of synaptic junction arise exclusively from aspinous and sparsely spinous stellate neurons (*13*), it appears likely that this neuronal type is the source of the GAD-positive terminals.

All of the monkeys that received alumina gel applications displayed frequent and chronic seizure activity as evidenced by EEG and ECG (Fig. 1) recordings.

Table 1. Mean number of GAD-positive axon terminals per 3000 μm^3 of tissue in layers V and VI of monkey sensorimotor cortex. Monkeys 1 and 2 received subarachnoid injections of alumina gel while the others had intracortical injections. Monkeys 1 to 3 were *Macaca mulatta* and monkeys 4 and 5 were *M. fascicularis*.

Animal	Proximal to alumina gel application	Ipsilateral cortex distal to alumina gel application	Nonepileptic contralateral cortex*
1	1.8	9.0	16.0
2	6.7	13.5	17.2
3	4.4	8.2	11.6
4	6.3	10.7	15.2
5	10.0	15.8	23.6
Overall mean of specimens	5.8	11.4	16.7

*The range of values observed in the homotopic, nonepileptic contralateral cortex was very similar to that observed in two hemispheres of a normal monkey (see text).

The areas around the sites of alumina gel application displayed the seizure focus, but the contralateral homotopic cortex lacked independent seizure activity (*12*). Low magnification, light microscopy of cortex from all seizure foci revealed staining intensitites for GAD that were lower than those of contralateral, nonepileptic cortices (Fig. 3). This variation in the overall intensity of GAD-positive staining between epileptic and nonepileptic cortex indicated a difference in the number of GAD-positive terminals present in these preparations; this difference was verified by quantitative analysis at higher magnification.

In addition to the specific immunocytochemical staining of GAD-containing axon terminals, preparations of epileptic cortex exhibited other peroxidase-dependent staining. When alumina gel was injected into the subarachnoid space, staining occurred in cortical layers I and II immediately beneath the gel; when the alumina gel was injected directly into the cortex, immunoperoxidase staining occurred in areas bordering on the alumina granuloma. These staining patterns were also observed in sections incubated in control serum, as well as in sections incubated in only the electron-donor and substrate reagents diaminobenzidine and hydrogen peroxide, respectively. At higher magnifications, this staining was observed to be due to ovoid structures that were much larger in size (3 to 5 μm) than GAD-positive terminals. Since these structures exhibited endogenous peroxidase-like activity and were located at sites where macrophages have been observed previously (*9*), it is likely that they represented peroxisomes within reactive macrophages.

Cortical layers V and VI were selected for determining the number of GAD-positive terminals because these layers did not display staining of peroxisomes in the sites chosen for counting. In addition, neurons that project to the spinal cord and other subcortical structures have their cell bodies within layers V and VI (*14*), and therefore an assessment of the numbers of GAD-positive terminals in these layers provides a measure for the relative amount of GABA-mediated synaptic inhibition of neurons that control motor behavior. For statistical purposes, the counts of GAD-positive terminals were averaged for layers V and VI because there were no significant differences between these two layers in either normal or epileptic cortex. For each of the five epileptic monkeys, the mean numbers of GAD-positive axon terminals per 3000 μm^3 at sites adjacent to alumina gel applications were at least 50 percent less than those in contralateral cortex (Table 1). The former sites correspond to seizure foci while the latter contralateral cortical sites lack independent spike activity (*12*). In addition, GAD-positive terminals were counted as ipsilateral sites located 0.5 to 1.0 cm away from the counting sites adjacent to alumina gel application. These more distal, ipsilateral sites also contained fewer GAD-positive terminals than contralateral cortex, and such regions are known to display persistent seizure activity after excision of intracortical alumina granulomas and subjacent cortex (*12*). An analysis of variance showed that highly significant differences occurred between the mean numbers of GAD-positive terminals within each of these cortical sites ($F = 59.67$, d.f. 2, 8; $P < .01$). Furthermore, individual comparisons of these data by the Newman-Keuls method (*15*) showed that the mean numbers of GAD-positive terminals in the primary epileptic sites were significantly less ($P < .01$) than those in both the more distal ipsilateral sites and contralateral cortex. Moreover, the mean numbers of GAD-positive terminals in more distal, ipsilateral sites were significantly less ($P < .01$) than those found in the contralateral cortex. Thus, the results of

these analyses indicate a highly significant decrease in the number of GABAergic axon terminals at sites of seizure foci.

The decreased number of GAD-positive terminals in our immunocytochemical preparations of epileptic monkey cortex could be explained by an alumina gel-produced loss of antigenicity of GAD molecules. However, this is unlikely since most of the alumina is located in macrophages with slight amounts in astrocytes (*16*). Furthermore, not all of the GAD-positive terminals are lost at seizure foci, and the staining of the remaining terminals indicates that the antigenicity of GAD is not affected by the alumina gel. In addition, a differential loss of antigenicity caused by differences in the diffusion of alumina from the application site is unlikely since, in monkeys with subarachnoid injections, the deep cortical layers display similar decreases of GAD-positive terminals to those observed in the superficial layers directly subjacent to the alumina gel. Therefore, the loss of immunocytochemically detectable GAD from cortical axon terminals indicates an actual loss of GAD molecules and this could be due either to a severe impairment of GAD synthesis, or to the degeneration of GABAergic somata or their axon terminals, or both.

Although previous biochemical data have indicated decreased GAD activities at seizure foci (*5*), our results extend this finding and show a numerical decrease of GAD-containing axon terminals. Whether these terminals actually degenerate or merely lose immunocytochemically detectable GAD is not known. However, a degeneration of GAD-containing terminals is suggested by the results of ultrastructural studies that show a decreased number of presumed inhibitory, symmetric synaptic junctions with somata and dendritic shafts of cortical neurons at seizure foci (*17*). In either event, a functional loss of GABAergic cortical neurons would occur. Our experimental preparations indicate that the magnitude of this loss is significant and could be expected to reduce the inhibitory synaptic control of pyramidal neurons, thus leading to a hypersensitivity of these cells to normal excitatory synaptic inputs (*4, 5*). The reason for this loss of GABAergic terminals at seizure foci is unknown, but it is possible that aspinous stellate neurons may be highly susceptible to alterations induced by alumina treatments. Further support for a GABAergic involvement in epilepsy is derived from pharmacological studies that show that certain convulsant and anticonvulsant drugs act at GABAergic synapses in the central nervous system (*18*). Thus, our results in combination with those of the other studies cited in this report support a hypothesis that a loss of functional GABAergic neurons leads to focal epilepsy.

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11. Fixative solutions contained 4 percent paraformaldehyde, 0.1 percent glutaraldehyde, and 0.002 percent $CaCl_2$ in a 0.12*M* phosphate buffer. Specimens for light microscopy were infiltrated with 30 percent sucrose solution, quickly frozen with Dry Ice, embedded in O.C.T. (Lab-Tek Products), and cut on a cryostat at a thickness of approximately 30 μm. Specimens for electron microscopy were sectioned on a Sorvall TC-2 tissue sectioner at a thickness of 150 μm and were rinsed in buffer overnight before being incubated in GAD immunochemical reagents. Sections were consecutively incubated for 1 hour in the following solutions; normal rat serum, rabbit antiserum to GAD or control rabbit serum, goat antiserum to rabbit immunoglobulin G. peroxidase-antiperoxidase Fab complex, and 3,3′-diaminobenzidine in 4*N* HCl (Sigma) and 0.006 percent H_2O_2. Sections were rinsed in buffer for 2.5 hours after each incubation. Sections were further processed for light and electron microscopic observations by established procedures. For more details, see R. P. Barber, J. E. Vaughn, K. Saito, B. J. McLaughlin, E. Roberts, *Brain Res.* **141**, 35 (1978); and (*6*) and (*7*).
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